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Survival of the recombinant *Bacteroides thetaiotaomicron* strain BTX in *in vitro* rumen incubations

M.A. Cotta, T.R. Whitehead and M.A. Rasmussen[1]

Fermentation Biochemistry Unit, National Centre for Agricultural Utilization Research, USDA, Agricultural Research Service, Peoria, IL, and [1]Enteric Diseases Research, National Animal Disease Centre, USDA, Agricultural Research Service, Ames, IA, USA

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M.A. COTTA, T.R. WHITEHEAD AND M.A. RASMUSSEN. 1997. The survival of *Bacteroides thetaiotaomicron* strain BTX under rumen-simulating conditions was studied. Strain BTX is a recombinant variant of strain 5482 engineered for the production of high levels of xylanase, an enzyme important in the degradation of hemicellulose. Strain BTX was not inhibited by compounds present in rumen fluid and it grew well in media containing rumen fluid (up to 75%) or high concentrations of volatile fatty acids (total concentration, 100 mmol l^{-1}). The ability of strain BTX to compete with other micro-organisms under rumen-like conditions was studied in *in vitro* incubations of rumen contents. These experiments employed a consecutive batch culture (CBC) system consisting of alfalfa suspended in a rumen fluid buffer inoculated with blended rumen contents and maintained by transfers (10%, v/v) at 48 h intervals. CBC cultures contained a diversity of microbial morphotypes and accumulated fermentation products in rumen-like proportions. When added alone, the numbers of BTX cells were maintained for only a few hours, and then declined precipitously until undetectable after 48 h. If CBC cultures were also supplemented with chondroitin sulphate (a mucopolysaccharide used by *Bact. thetaiotaomicron*), strain BTX grew and the pattern of its population generally followed that of the total population of ruminal bacteria in these cultures. When transferred into fresh CBC cultures containing chondroitin sulphate, BTX was again able to grow and increase in numbers, but to a diminished degree. Although BTX was able to survive and maintain itself in chondroitin sulphate supplemented cultures, this was at a very low level (10^5 ml^{-1}). The potential for manipulation of rumen function by inoculation with recombinant bacteria is discussed.

INTRODUCTION

Microbial digestion and fermentation in the rumen enables ruminant animals (sheep, cattle, goats, etc.) to subsist on diets composed largely of fibrous plant materials. These plants contain large quantities of cellulose and hemicellulose which cannot be hydrolysed by the host's mammalian digestive enzymes. While this confers an advantage to these animals and allows them to convert relatively inexpensive feeds into high quality animal products, the utilization of these complex polysaccharides is often incomplete. As a result, higher quality and more costly feeds are added to diets to meet the nutrient demands of high levels of meat and milk production of modern livestock production systems. Efforts to improve the utilization of the fibre portion of animal diets have centred on physical and chemical treatments of forage materials. Grinding, pelleting, steam explosion of forages, or treatments with alkali, peroxides and other chemicals have been used to increase the susceptibility of plant cell walls to microbial

Correspondence to: Dr Michael A. Cotta, Fermentation Biochemistry Unit, National Centre for Agricultural Utilization Research, USDA, Agricultural Research Service, 1815 N. University Street, Peoria, IL 61604, USA (e-mail: cottama@ncaurl.ncaur.gov).*

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colonization and enzymatic attack (Fahey *et al.* 1993). An alternative to this approach would be to try to modify the activities of the micro-organisms themselves to increase their capacity for digestion of these materials.

The development of molecular biological techniques has offered a means of genetically manipulating micro-organisms for specific purposes. This approach has been applied successfully in a variety of industrial settings (e.g. production of pharmaceuticals), and illustrates the potential for modification of ruminal bacteria to increase their ability to degrade fibrous feedstuffs (Smith and Hespell 1983; Russell and Wilson 1988; Flores 1989; Wallace 1992; Forsberg *et al.* 1993). Initial attempts in this laboratory to genetically manipulate ruminal bacteria focused on improving the xylanolytic activity of *Prevotella ruminicola* (formerly *Bacteroides ruminicola*). A xylanase gene from *P. ruminicola* strain 23 was successfully cloned into *Escherichia coli*, sequenced and characterized (Whitehead and Hespell 1989; Whitehead 1993). However, efforts to reintroduce this gene into *P. ruminicola* by transformation, electroporation and conjugation to increase its production of xylanase failed. The reasons for this failure are unknown, but may be due in part to the activity of restriction-modification enzymes which this species is known to possess (M. Simcox, Stratagene Cloning Systems, La Jolla, CA; personal communication). In contrast, this gene was highly expressed when introduced into closely related human colonic bacteroides species (Whitehead and Hespell 1990). Subsequently, it was demonstrated that the gene could be stably maintained in the absence of antibiotics if the gene was inserted into the chromosome of another human colonic species, *Bacteroides thetaiotaomicron* (Whitehead *et al.* 1991). The resultant organism, *Bact. thetaiotaomicron* strain BTX, produced xylanase at elevated levels and degraded oat spelt xylan in culture. Strain BTX was unable to ferment the oligosaccharide products of xylan hydrolysis, but these carbohydrates could be utilized by oligosaccharide fermenting ruminal bacteria in coculture (Cotta and Whitehead 1991). Although *Bact. thetaiotaomicron* is not of rumen origin, it is physiologically quite similar to many rumen bacteria. That is, it is an anaerobe capable of growing on a variety of carbohydrate sources, it grows within the temperature and pH ranges common to the rumen, and it is resistant to the concentrations of salts and other ions present in the rumen (Holdeman *et al.* 1977; Dowell and Lombard 1981; Macy 1981). In addition, the natural environment inhabited by this organism (i.e. human colon) is a complex microbial ecosystem, so *Bact. thetaiotaomicron* must have adapted mechanisms to compete for available substrates with other organisms present. Based on this, it appears that strain BTX could possibly survive in the rumen. The objective of the current study was to examine whether strain BTX could indeed survive under *in vitro* rumen-like conditions. The information obtained will be helpful in establishing whether further development of this strain as a rumen inoculum is warranted or what additional conditions need to be fulfilled before successful inoculation into ruminant animals can be attempted.

MATERIALS AND METHODS

Organisms

Bacteroides thetaiotaomicron strain 5482 and its recombinant derivative, strain BTX, were used in these experiments. Strain BTX is strain 5482 to which the xylanase gene from *Prevotella ruminicola* strain 23 along with a clindamycin resistance gene has been incorporated into the chromosome, yielding a recombinant strain that produces high levels of xylanase activity. The construction of strain BTX is described in detail elsewhere (Whitehead *et al.* 1991). These strains were routinely cultivated in a complex yeast extract–trypticase–salts medium (RGM, Hespell *et al.* 1987). Maintenance medium for strain BTX also contained 10 $\mu g\,ml^{-1}$ clindamycin to ensure maintenance of the xylanase and clindamycin resistance genes. Before BTX was used in any experiments, it was passed through three transfers (2% inoculum) in clindamycin-free medium to minimize the carryover of antibiotic into experimental media. Growth was monitored spectrophotometrically by measuring optical densities of cultures at 660 nm (Spectronic 21, Milton Roy Company, Rochester, NY).

Consecutive batch cultures

A consecutive batch culture (CBC) system was constructed to study the survival of strain BTX under rumen simulating conditions. This *in vitro* rumen incubation system was modelled after that described by Theodorou and coworkers (Theodorou *et al.* 1984, 1987; Gascoyne 1986; Gascoyne *et al.* 1988) and consisted of 18 × 150 mm serum stoppered tubes into which 100 mg of ground alfalfa (3 mm screen) and 9 ml of 30% clarified rumen fluid in an anaerobic buffer (RGM medium without added trypticase, yeast extract and volatile fatty acids) were dispensed. CBCs were inoculated with 1 ml of strained, blended rumen contents collected from a ruminally fistulated steer fed a diet of alfalfa hay. Cultures were incubated at 39°C and maintained by 10% (v/v) transfer of the contents at 48 h intervals. *Bacteroides thetaiotaomicron* BTX was inoculated into CBC cultures on the fourth transfer. Chondroitin sulphate was added to some experimental cultures to provide an additional energy source for growth of strain BTX. *Bacteroides thetaiotaomicron* is able to grow on this mucopolysaccharide and insertional mutagenesis of the chondroitin lyase II gene does not impair the ability of this organism to grow on this substrate (Guthrie and Salyers 1986). Enumerations of chondroitin sulphate fermenting bac-

teria in the rumen inoculum indicate the population of these organisms added to CBC cultures was low (less than 10^3 ml^{-1}).

Experimental treatments included cultures inoculated with $\approx 1 \times 10^8$ BTX cells ml^{-1}, 5×10^7 BTX cells ml^{-1} and 0·05% chondroitin sulphate, and controls without added BTX or chondroitin sulphate. The number of BTX cells present in the inoculum was estimated from a previously determined optical density to viable cell count standard curve for growth in the medium employed in these experiments. All experiments were performed in triplicate. CBC cultures were routinely sampled after 48 h of growth, but also at 3, 6, 9, 12, 24 and 33 h after inoculation with strain BTX. Cultures were analysed for methane production, organic acids, pH, total bacterial numbers and *Bact. thetaiotaomicron* BTX numbers. On the fourth, fifth, sixth and ninth transfers, additional parallel cultures (in triplicate) were inoculated and after 48 h the entire cultures were used for determinations of dry matter disappearance, xylose and arabinose utilization.

Table 1 Selective medium for enumeration of *Bacteroides thetaiotaomicron* strain BTX

Component	Amount per 100 ml
50 mmol l^{-1} sodium phosphate	90 ml
Macrominerals*	10 ml
Trace minerals†	1 ml
Vitamin B_{12}	0·5 μg
Ammonium chloride	0·05 g
Hemin	0·1 mg
Cysteine HCl	0·05 g
Chondroitin sulphate	0·2 g
Agar	1·8 g
Clindamycin	1 mg

* Macromineral solution contained (g l^{-1}): KH_2PO_4, 6·8; NaCl, 5·0; $(NH_4)_2SO_4$, 3·3; $MgSO_4 . 7H_2O$, 1·0; $CaCl_2 . 2H_2O$, 0·7.
† Trace mineral solution contained (mg l^{-1}): Na_2EDTA, 500; $FeSO_4 . 7H_2O$, 200; $MnCl_2 . 4H_2O$, 200; $ZnSO_4 . 7H_2O$, 10; H_3BO_4, 30; $CoCl_2 . 6H_2O$, 20; $CuCl_2 . 2H_2O$, 1; $NiCl_2 . 6H_2O$, 2; $NaMoO_4 . 2H_2O$, 3.

Bacterial enumerations

Total bacterial numbers were determined by plating appropriately diluted culture samples onto a non-selective medium in an anaerobic glove box (75% nitrogen, 20% carbon dioxide, 5% hydrogen; Coy Laboratory Products, Ann Arbor, MI). The non-selective medium employed was based on the RGM medium supplemented with 30% clarified (6000 *g*, 30 min) rumen fluid, 1·8% agar, and with 0·1% each of glucose, maltose, xylose and cellobiose as carbohydrates. Dilutions were performed anaerobically in the same medium but without agar and carbohydrates. Plates were incubated at 37°C.

The numbers of *Bact. thetaiotaomicron* were estimated using a selective medium containing chondroitin sulphate and clindamycin (Table 1). Dilutions were performed in a dilution buffer composed of macrominerals, microminerals and water (Table 1) and prepared without precaution to exclude oxygen. Plates were inoculated under atmospheric oxygen conditions and then transferred to an incubator (37°C) in the anaerobic chamber for growth. These manipulations were performed in this manner to take advantage of the tolerance exhibited by *Bact. thetaiotaomicron* toward exposure to oxygen and reduce the potential for growth of non-BTX cells in this diagnostic medium. Preliminary experiments demonstrated that BTX could be manipulated in this way without loss of enumeration efficiency. The presence of the xylanase character in presumptive BTX clones was confirmed by randomly picking (at least 100) colonies and transferring them to selective medium overlaid with remazol brilliant blue-Xylan (Whitehead and Hespell 1989). Zones of clearing indicated a positive reaction for the production of xylanase activity. All presumptive BTX clones determined by this method were xylanase positive.

Chromatographic methods

The neutral sugar components of samples were determined by gas-liquid chromatography of alditol acetates prepared by the procedure of York *et al.* (1985) as modified by Stack (1987). Samples (10 mg) were hydrolysed in trifluoroacetic acid, reduced with sodium borohydride, and acetylated with acetic anhydride. Inositol was added as an internal standard. The resultant alditol acetates were resolved on a DB-225 fused silica capillary column (J&W Scientific, Rancho Cordova, CA) heated to 210°C in a Hewlett-Packard Model 5890 A gas chromatograph (Hewlett-Packard, San Fernando, CA) and identified by comparison with the retention times of authentic standards.

The concentrations of fermentation end products present in samples were determined by GLC of butyl esters of organic acids prepared by the procedure of Salanitro and Muirhead (1975). Heptanoic acid was added as an internal standard. The resultant butyl esters were resolved on a DB-225 fused silica capillary column (J&W Scientific, Rancho Cordova, CA) heated to 210°C in a Hewlett-Packard Model 5890 A gas chromatograph and identified by comparison with the retention times of authentic standards.

Gaseous products present in the headspace of CBC cultures were resolved on a Carbosieve G column (Supleco, Bellefonte, PA) heated to 170°C in a Hewlett-Packard Model 5890 A gas chromatograph, detected by thermal conductivity,

and identified by comparison with the retention times of authentic standards. Argon was the carrier gas (10 ml min^{-1}).

Dry matter utilization

Cultures frozen for dry matter disappearance determinations were thawed and the entire contents transferred to centrifuge tubes. Samples were centrifuged (10 000 *g*, 4°C, 20 min), the supernatant fluid was decanted, and the pellets were suspended in water, and then centrifuged again. The supernatant fluid was again discarded, and the washed pellets were transferred to preweighed aluminium pans, frozen and lyophilized to dryness. Dry matter disappearance was determined as the difference between the amount of alfalfa added to each CBC culture tube and the mass recovered as freeze-dried residue. Dry matter disappearance determined in this manner represents a net loss since no effort was made to account for the gain in microbial biomass contained in residues.

Chemicals

Chondroitin sulphate A was obtained from Sigma Chemical Co. (St Louis, MO). All chemicals used were of the highest grade available.

RESULTS

Characteristics of strain BTX

Survival in presence of volatile fatty acids. The ability of *Bact. thetaiotaomicon* strain BTX to grow in media containing rumen fluid was examined. Neither the growth rate nor the growth yield (final optical density) appeared to be affected by the presence of up to 75% rumen fluid in RGM medium (Fig. 1). Similarly, the addition of 10 times the normal supplement of volatile fatty acids (i.e. acetic, propionic, butyric and branched-chain acids in rumen-like proportions) to RGM medium did not inhibit the growth of strain BTX (not shown). This was equivalent to a total organic acid concentration of greater than 100 mmol l^{-1}.

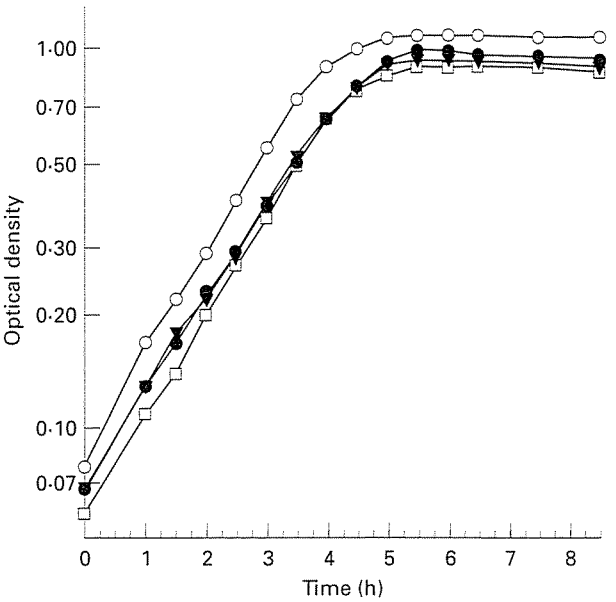

Fig. 1 Growth of *Bacteroides thetaiotaomicron* strain BTX in rumen-fluid containing medium. Strain BTX was grown in RGM medium adjusted to contain (v/v): ●, 0% rumen fluid; ○, 25% rumen fluid; ▼, 50% rumen fluid; □, 75% rumen fluid

Table 2 Competition of recombinant strain BTX with parental *Bacteroides thetaiotaomicron* strain 5482 in batch culture*

	Cell numbers ($\times 10^7$)		Per cent	
Days	BTX	5482	BTX	5482
0	0·13	0·22	37	63
1	540	100	83	17
2	640	ND	100	0
3	700	ND	100	0
4	620	70	90	10
5	1000	20	98	2
7	1000	80	93	7

* Cultures were transferred daily into fresh RGM medium containing 0·2% glucose.
ND, Not detectable, less than 1×10^7.

Competition between BTX and 5482. The results of experiments examining the competition between strain BTX and the parental strain, 5482, are shown in Table 2. Similar amounts of each strain were inoculated into RGM medium with glucose as a carbohydrate source and the cultures were transferred into fresh medium daily. The numbers of each strain were estimated by determining the difference between plate counts on selective medium (Table 1) with and without added clindamycin. Although initially present at slightly lower numbers, strain BTX quickly became dominant, comprising 100% of the detectable cells within three transfers. Ultimately the numbers of the parental strain 5482 increased again, and appeared to reach equilibrium at ≈10% of the culture population by the seventh transfer. The strains exhibited slightly different maximal growth rates in glucose-supplemented RGM medium with strain BTX always growing more rapidly ($\mu = 0{\cdot}52\ h^{-1}$ *vs* $\mu = 0{\cdot}46\ h^{-1}$).

Characteristics of CBC fermentations

CBC cultures were initiated by inoculation with rumen contents to $\approx 1 \times 10^8$ bacteria ml^{-1} of CBC culture and main-

Table 3 Characteristics of consecutive batch cultures

	Control	Plus BTX	Plus BTX and chondroitin sulphate
pH	6·39 ± 0·06	6·38 ± 0·05	6·38 ± 0·07
Fermentation end products:			
Total (mmol l^{-1})	57·11 ± 6·48	54·87 ± 7·37	57·98 ± 5·63
Molar proportions (%):			
acetic acid	71·4 ± 1·3	71·2 ± 1·4	71·8 ± 1·7
propionic acid	21·5 ± 1·4	21·7 ± 1·3	21·4 ± 1·3
butyric acid	7·1 ± 0·6	7·1 ± 0·5	6·8 ± 0·7
Other products:			
lactic acid		never detected	
succinic acid		never detected	
methane		always produced	
hydrogen		never detected	
Digestion parameters:			
Dry matter disappearance (%)	44·70 ± 4·77	45·62 ± 4·57	46·36 ± 5·81
Microscopic observations:			
	Diversity of bacterial morphologies present. Protozoa rapidly lost: not present after second transfer		

tained by transfers of 10% of the culture contents into fresh medium at 48 h intervals. Based on the concentration and composition of end products formed, these cultures stabilized within the first two transfers. These incubations always contained a diversity of bacterial morphotypes and the major microbiological change observed was the rapid loss of protozoa from these cultures. Values for the various parameters monitored did not vary greatly for each 48 h sampling and the results presented in Table 3 represent values averaged for the entire experimental period. CBC cultures accumulated major fermentation end products in the proportions shown in Table 3. Lactic acid, succinic acid and hydrogen were not detected and methane was always produced. Cultures utilized slightly less than half of the alfalfa substrate and the final pH of fermentations was about 6·4. An attempt was made to estimate the xylan utilization by CBC cultures by monitoring the concentrations of xylose and arabinose present in residue remaining after 48 h of growth, but the results were extremely variable. The utilization of xylose by all cultures averaged about 16% and about 45% of the arabinose present disappeared. The addition of strain BTX, with or without added chondroitin sulphate (0·05%), resulted in little alteration in any of the fermentation parameters measured.

Survival of strain BTX in CBC cultures

After the CBC cultures had stabilized (three transfers), two sets of cultures were inoculated with strain BTX (with and without added chondroitin sulphate), while a third set received no additions (control). The numbers of BTX cells and the total bacterial population present were measured from the time of BTX addition until the end of the experiment. The total bacterial numbers increased during the first 12 h following inoculation, but this number had declined by 24 h (Fig. 2). There was a slight increase in the numbers present at 30 h, before declining again at 48 h. The numbers present at each subsequent 48 h interval (before transfer)

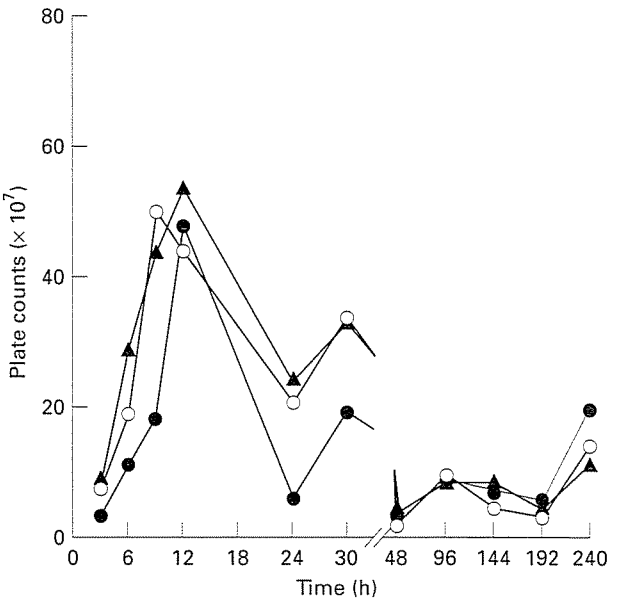

Fig. 2 Counts of total culturable bacteria in CBC incubations following inoculation of cultures with *Bacteroides thetaiotaomicron* BTX. ○, Cultures inoculated with BTX; ▲, cultures inoculated with BTX and 0·05% chondroitin sulphate; ●, control cultures receiving no additions

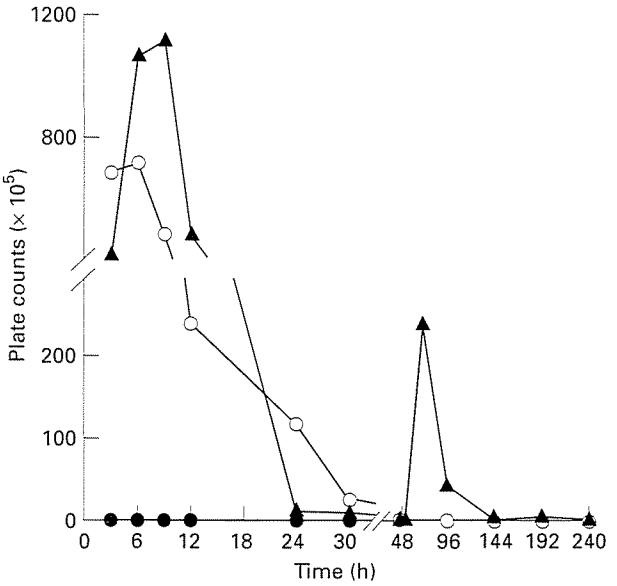

Fig. 3 Counts of *Bacteroides thetaiotaomicron* BTX in consecutive batch culture incubations following inoculation of cultures with *Bact. thetaiotaomicron* BTX. ○, Cultures inoculated with BTX; ▲, cultures inoculated with BTX and 0·05% chondroitin sulphate; ●, control cultures receiving no additions

were fairly constant. The numbers present in the different experimental cultures exhibited similar patterns regardless of the addition of BTX and chondroitin sulphate, although the BTX-containing cultures tended to be slightly higher at 30 h.

The numbers of BTX in CBC cultures were determined using a minimal chondroitin sulphate, clindamycin-amended medium (Fig. 3). When strain BTX was inoculated into CBC cultures at $\approx 1 \times 10^8$, the numbers of BTX cells remained steady for the first 9 h of incubation then declined over the next 37 h. Low levels of BTX cells were present at $\approx 2 \times 10^5$ after 48 h and were not detected in subsequent transfers. In contrast, BTX cells increased to $\approx 1 \times 10^8$ over the first 12 h when added at 5×10^7 and supplemented with chondroitin sulphate to promote growth. These numbers then rapidly declined to $\approx 1 \times 10^6$ by 24 h of incubation. The numbers of BTX cells present in these cultures were also $\approx 2 \times 10^5$ after 48 h of incubation. When transferred to fresh medium containing chondroitin sulphate, the numbers of BTX again increased to $\approx 2 \times 10^7$ by 72 h (24 h after second transfer). BTX cells were detected in 48 h samples of all subsequent transfers but only at $\approx 2 \times 10^5$. Presumptive BTX cells were not detected in the control incubations at any sampling time (less that 10^3).

Since strain BTX appeared capable of growth in incubations supplemented with chondroitin sulphate, a second CBC experiment examining the survival of strain BTX was conducted (not shown). In these experiments, the numbers of BTX were determined at 24 h in addition to 48 h after each transfer. The 24 h sampling time was selected since it was estimated as the time required for 10^5 cells of strain BTX to grow and completely use the chondroitin sulphate provided. The results of this experiment confirmed those displayed in Fig. 3. As before, the numbers of BTX increased following transfer and then declined to barely detectable by 2 d of incubation. Interestingly, the peak in BTX numbers decreased with each additional transfer.

DISCUSSION

In recent years, several research groups have attempted to genetically alter the activities of various ruminal bacteria (Gobius *et al.* 1995; Gregg 1995; Whitehead and Flint 1995; Gardner *et al.* 1996). This has been done primarily to provide a clearer understanding of the biochemical and physiological activities of these organisms at the molecular level. However, this research also explored the potential for use of microbial inocula to beneficially modify rumen fermentation. Similarly, a number of non-ruminal microbes have been proposed as feed additives to improve ruminal digestion (Dawson *et al.* 1990; Muirhead 1992; Wallace 1994; Newbold *et al.* 1995). In either case, the inoculated cells must reside in the rumen long enough to produce the desired effect on rumen function. Most often this will require that the organism compete with ruminal microbes present for growth substrates available. In addition, they must also be resistant to the physical and chemical conditions present in the rumen.

Bacteroides thetaiotaomicron strain BTX was constructed to produce xylanase, an enzyme that could increase the digestion of hemicellulose in the rumen (Whitehead *et al.* 1991). The xylanase gene was introduced into the chondroitin lyase II gene in the chromosome where it was stably maintained without antibiotic selection. Guthrie and Salyers (1986) showed that the chondroitin lyase II could be disrupted in this way without affecting the organism's ability to grow on chondroitin sulphate or its ability to colonize the intestinal tract of mice (Salyers and Guthrie 1988). Our results confirm their findings and demonstrate that not only is growth unimpaired, but may provide a slight growth rate advantage over the parental strain. These findings, coupled with the ability of strain BTX to grow in the presence of high concentrations of volatile fatty acids (Fig. 1), encouraged us to examine whether this recombinant strain could grow and compete with other ruminal bacteria in simple *in vitro* rumen fermentations.

A consecutive batch culture (CBC) system was used for these experiments. The chemical and microbiological characteristics of CBC cultures were similar to those reported by others using comparable batch incubations of rumen contents (Leedle and Hespell 1983; Argyle and Hespell 1987). In our experiments, cultures accumulated fermentation products in rumen-like proportions, methane was always produced with no detection of lactate, succinate and hydrogen. Aside from

the loss of protozoa, this model served our needs well for initial survival experiments.

Strain BTX did not survive long in CBC incubations, indicating it was unable to compete with other bacteria present for carbohydrates generated during the digestion of alfalfa. Therefore, chondroitin sulphate was added as a supplement to promote the growth of BTX. *Bacteroides thetaiotaomicron* grows well on chondroitin sulphate and preliminary experiments indicated that the numbers of chondroitin sulphate fermenting organisms in the rumen are low (less than 10^3). Strain BTX grew and persisted in chondroitin supplemented incubations, but its numbers declined with each transfer until they were barely detected after a few transfers. It seems possible that, although initially low in numbers, a population of chondroitin sulphate fermenting microbes were able to out-compete BTX for this substrate and quickly dominate. Alternatively, the CBC culture system used in our experiments may not include a metabolic niche for strain BTX that exists in the rumen environment. Preliminary results using dual flow continuous cultures, a more sophisticated rumen simulation technique (Hoover *et al.* 1976; Mansfield *et al.* 1994), indicate strain BTX may survive and persist under rumen-like conditions even without added chondroitin sulphate. This rumen model allows for the provision of more complex diets (than alfalfa) and achieves culture turnover rates that closely mimic those observed *in vivo*.

The evidence from a variety of sources indicates that the likelihood of successfully introducing a bacterium not of rumen origin into the rumen is low (Russell and Wilson 1988; Wallace *et al.* 1989; Wallace 1994). These discussions have focused primarily on the potential for using genetically modified strains of *E. coli*. The current work extends this and suggests that even organisms derived from ecosystems similar to the rumen and apparently well adapted to survive in the rumen may not be able to compete against the normal rumen microflora. Our understanding of the molecular biology of ruminal anaerobes is gradually improving and the types of manipulations that can be performed are expanding. Recently, a few workers have had some success inoculating the rumen with modified strains of ruminal bacteria (Flint *et al.* 1989; Brooker and Lum 1993; Gregg 1995). Future research should continue to focus on the development of genetic tools required to manipulate authentic ruminal bacterial species.

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